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Flow cytometric analysis of virus-like particles and heterotrophic bacteria within coral-associated reef water

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Using flow cytometry, two distinct populations of virus-like particles (VLP) and heterotrophic bacteria were defined within the 12 cm water layer immediately overlying healthy, diseased and dead acroporid corals. Bacterial abundances were similar in overlying water for all coral types, however, VLP were 30% higher above diseased corals than healthy or dead corals. Mean virus to bacteria ratios (VBR) were up to 30% higher above diseased corals than above healthy or dead coral or in distant water. Concomitant with increasing VLP concentrations within 5 cm of coral surfaces, VBR distributions were generally highest above healthy and diseased coral and depressed above dead coral. These results suggest fundamental shifts in the VLP and bacterial community in water associated with diseased corals.

INTRODUCTION

Corals harbour an abundant, diverse and dynamic consortium of microbial communities within the coral surface microlayer, the layer extending a few millimetres from the coral surface, and within coral tissue (Ducklow & Mitchell, 1979; Paul et al., 1986, Rohwer et al., 2001, 2002; Kellogg, 2004; Bourne & Munn, 2005). Viruses, while not routinely studied in coral–microbial associations, represent a potentially important yet overlooked community involved in the overall health of corals (Seymour et al., 2005). Marine viruses are known to primarily infect prokaryotes (Proctor & Fuhrman, 1990) and eukaryotic phytoplankton (Suttle et al., 1991), however, viruses have also been shown to infect cnidarians and symbiotic zooxanthellae (Wilson & Chapman, 2001; Wilson et al., 2001, 2005). Subsequently, viral infection has been proposed as a mechanism involved in coral-bleaching events (Wilson et al., 2001) but, as yet, there have been no reports of viral associations with bleached or diseased corals in natural systems.

One way in which we can detect the presence of coral-related viruses and bacteria is to compare water immediately overlying coral with those more distant water samples (van Duyl & Gast, 2001). The influence that corals have on overlying waters is likely to be most pronounced in the few centimetres of water immediately above corals (van Duyl & Gast, 2001). Beyond a few centimetres, mixing processes will dilute any signal (e.g. mucus, nutrients, associated bacteria, viruses, phytoplankton and zooxanthellae) exuded from the coral (Gast et al., 1998). Indeed, water column bacteria populations appear to be dissimilar to coral-associated bacteria (Rohwer et al., 2001). We have recently demonstrated that significantly higher abundances of virus-like particles (VLP) occur within the few centimetres of water closest to the coral surface (Seymour et al., 2005). Here, we present evidence for shifts in VLP and bacteria in water immediately overlying healthy, diseased and dead branching acroporid corals in the Great Barrier Reef.

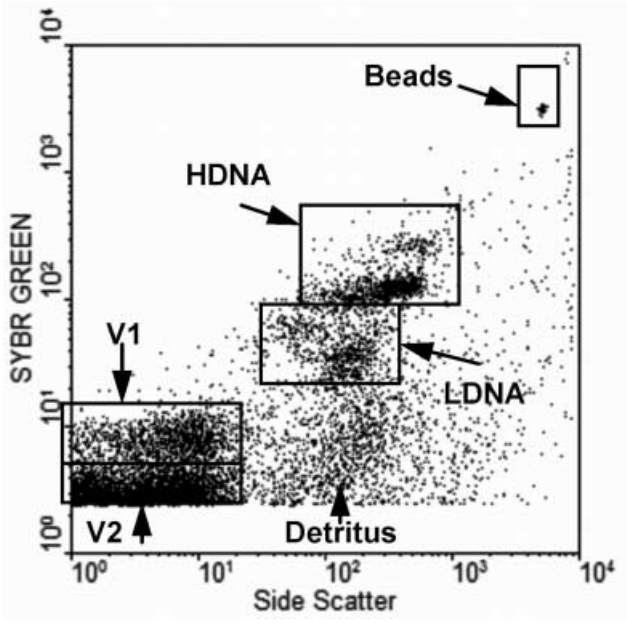

Figure 1. A characteristic cytogram illustrating flow cytometrically-defined bacterial and virus-like particles (VLP) populations from coral-water immediately above a healthy coral (*Acropora* spp.) at Myrmidon Reef. Two VLP populations, virus population 1 (V1) and virus population 2 (V2) and two bacterial sub-populations representing high (HDNA) and low DNA (LDNA) groups can be discriminated. All flow cytometric parameters were normalized to concentrations of 1 μm diameter fluorescent beads (Molecular Probes).

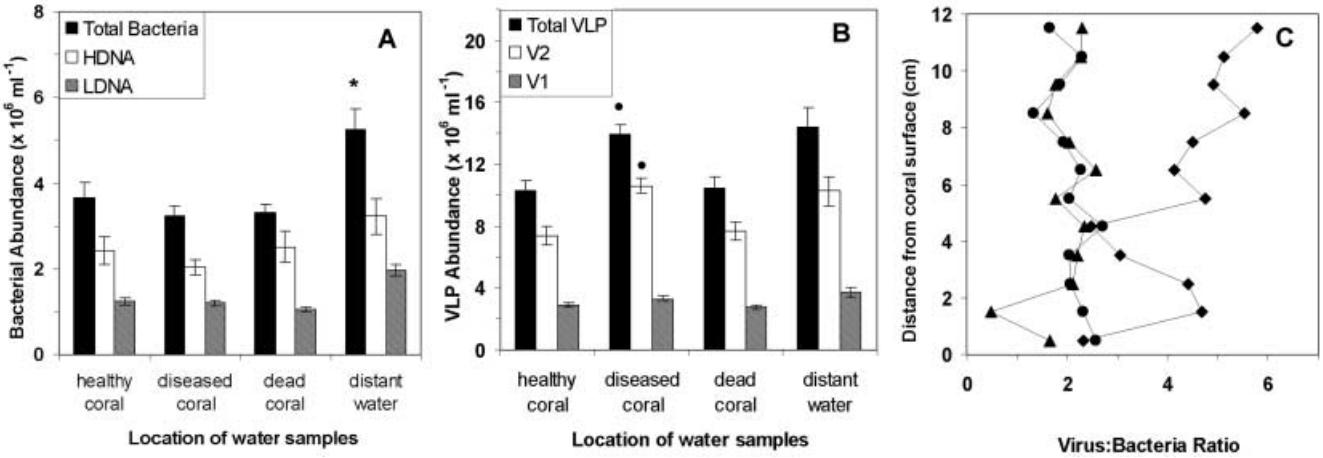

Figure 2. (A) Comparisons between total bacteria, high DNA (HDNA) and low DNA (LDNA) groups; and (B) comparisons between total virus-like particles (VLP), virus population 1 (V1) and virus population 2 (V2) in coral water above healthy, diseased and dead coral (*Acropora* spp.) and in 1 m distant water. One-way analysis of variance (ANOVA) and post-hoc Tukey tests were used to determine differences between coral-water sampled above healthy (N=36), diseased (N=24) and dead coral (N=36) and with 1 m distant water samples (N=24). Errors are means $\pm$95% confidence limits. * indicates values are significantly different from all other water types ($P<0.05$) and ● indicates values are significantly different from all other coral-water ($P<0.05$). (C) Examples of virus to bacteria ratios (VBR) within the 12 cm water layer overlying a healthy (●), diseased (◆) and dead (▲) branching coral.

MATERIALS AND METHODS

A linear profiling device consisting of 12 sterile 1 ml syringes each separated by a distance of 10 mm was employed to sample the 12 cm water layer (termed 'coral-water') (Seymour et al., 2005) immediately overlying three healthy, two white syndrome diseased and three dead branching acroporid corals at Myrmidon Reef (147°22′E 181°6′S), an outer exposed reef situated in the Great Barrier Reef Marine Park. Healthy corals exhibited strong pigment and were devoid of obvious abrasions. White syndrome disease was easily identified and characterized by coral colonies exhibiting distinct separation between the healthy coral tissue and white exposed skeleton (Willis et al., 2004). Dead corals were identified as coral skeletons devoid of tissue and colonized by thin ($\sim$2 mm) algal turf mats. All profiles were sampled in water directly above the surface of coral colonies ensuring that the distance between the first syringe in the linear array and the top coral surface was $\sim$0.5 cm. Profiles above diseased corals were sampled with the profiler positioned at the interface of the healthy coral tissue and the white skeleton. Individual 1 ml samples were fixed in glutaraldehyde (0.5% final concentration) for 20 min in the dark, frozen in liquid nitrogen and stored at -80°C (Marie et al., 1999). The spatial distribution of VLP and heterotrophic bacteria within coral-water was compared with 1 m distant water samples using flow cytometric analysis according to the methods of Seymour et al. (2005) adapted from Marie et al. (1999).

The limit of background noise detection in the flow cytometric analysis was determined using SYBR I Green stained 0.02 μm sterile seawater and TE buffer (10 mM Tris, 1 mM EDTA, [pH 7.5]). The threshold for the flow cytometer was set to ensure noise present in control samples was removed from the analysis. Virus-like particles were then defined as SYBR Green fluorescent particles above background noise but having lower fluorescence levels than bacterial populations (see figure 1 in Seymour et al., 2005). Unlike the presence of only one distinguishable VLP population in a similar study (Seymour et al., 2005), we identified two VLP populations in cytograms, virus population 1 (V1) and virus population 2 (V2) (Figure 1). There is the possibility that a small proportion of V2 counts may be associated with noise artefacts, as on some occasions we observed a slight overlap between the V2 population and background noise. However, background noise accounted for no more than 5% of recorded events within the V2 population. To reduce the possibility of including some erroneous noise measurements within the V2 population, V2 counts were always obtained by correcting noise with stained 0.02 μm seawater and TE buffer. Alternatively, and a more likely scenario, however, is that the abundance of the V2 population may be a slight underestimate of total abundance due to the strict SYBR I Green threshold applied.

RESULTS AND DISCUSSION

Based on differences in SYBR Green fluorescence (DNA content) and side scatter (an indicator of cell size), heterotrophic bacteria were divided into two distinct populations, high DNA (HDNA) and low DNA (LDNA) (Gasol et al., 1999; Lebaron et al., 2001, 2002) (Figure 1). It has previously been demonstrated that HDNA cells represent the active component of bacterioplankton communities, while LDNA cells are likely to consist of dormant or dead bacteria (Gasol et al., 1999; Lebaron et al., 2001, 2002).

Water-column bacterial communities have been shown to be dissimilar from coral-associated bacterial communities (Rohwer et al., 2001; Frias-Lopez et al., 2002). Here, the total mean bacterial concentration was significantly higher in 1 m distant samples than in all coral-water (all $P<0.05$) (Figure 2A). However, by employing the percentage of HDNA bacterial index (%HDNA)

(Gasol et al., 1999), active bacterial cells were 10% higher in coral-water compared with 1 m distant water (all $P<0.05$). These results differ from those in our previous study (Seymour et al., 2005). The reasons for this are unclear, however, a higher proportion of active cells within close proximity to coral substrates lend support to other workers who have shown elevated bacterial growth (Gast et al., 1998; Ferrier-Pagès et al., 2000; van Duyl & Gast, 2001) and productivity in waters overlying coral substrates (Moriarty et al., 1985).

There was evidence to suggest partitioning of viral populations between coral-water from corals differing in their health status. While V1 concentrations appeared similar for coral-water sampled above healthy, diseased and dead corals, V2 concentrations were 30% higher in coral-water above diseased coral than for healthy or dead coral (all $P<0.02$) (Figure 2B). Considering that bacterial abundances were similar between all coral types, these results indicate an uncoupling of bacteria and VLP populations within the waters overlying diseased corals. It is important to note however, that both V1 and V2 concentrations within coral-water immediately above diseased coral did not differ significantly from those in 1 m distant water samples (Figure 2B). Further studies are required to determine whether the taxonomic composition of the viral communities vary between distant water and coral-associated reef water, between different coral species and or between corals which differ in their health status. Indeed, coral-associated bacteria display species-specificity (Rohwer et al., 2001) and distinct partitioning between healthy and diseased coral species has been shown (Frias-Lopez et al., 2002). Consequently, we are currently developing suitable methods for coral-associated viral genomic extraction and subsequent sequencing to explore viral diversity in coral reef ecosystems.

Further information regarding the relationship of bacteria and VLP was provided with the virus to bacteria ratio (VBR) (Wommack & Colwell, 2001). Profiles of VBR also revealed that concomitant with 'peaks' in VLP concentrations occurring within the closest 5 cm to the coral surface that VBR were almost always highest in the first few centimetres of coral-water overlying healthy and diseased coral and depressed in the few centimetres overlying the dead coral–algal surface (Figure 2C). Furthermore, mean VBR was up to 30%-fold higher in coral-water above diseased coral than above healthy or dead coral or in distant water (all $P<0.02$), with no significant differences between any other coral-water samples. The VBR was lowest in distant water samples further suggesting that while similar abundances of VLP occur within distant water and coral-water associated with diseased coral, that partitioning of microbial populations may occur between these water types.

Stress induced changes in the coral holobiont (the coral, zooxanthellae and associated microbes inclusive) have been hypothesized to be important in coral health (Rohwer et al., 2002). While our sample sizes were small, differences in VLP dynamics above diseased corals, as observed here, could be a consequence of changes to the normal bacterial associations of healthy corals from unknown stressors (Rohwer & Kelly, 2004) or, alternatively, that VLP populations play a direct role in coral disease. Given the limited understanding of the role of microbes in coral diseases, the involvement of viruses in coral reef pathology represents a potentially important area of research.

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